Design, development and characterization of biosensors and nanoparticulate carriers for renal cell carcinoma

SUMMARY FOR THE DEGREE OF

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BACKGROUND OF THE STUDY

Renal cell cancer ranks in the top-ten list of malignancies both in males and females and its frequency is increased due to large aging population and obesity globally [1]. Renal cell cancer comprises of a group of tumors arising from renal tubular epithelial cells with an incidence rate of about 5–10 cases per 100,000 people and accounts for 2–3% of all malignant tumors [2]. Although chemotherapy is one of the main approaches for cancer treatment but its effectiveness is limited due to the development of drug resistance [3]. Targeted nanoparticles possess various advantages in cancer therapy with the enhancement of selectivity of drugs to the cancerous cells for avoidance of side effects to normal cells, improvement of drug accumulation and promotion of selective antineoplastic action on infected cells.

Silibinin (*Silybum marianum*) is a bioactive component obtained from the seeds of 'milk thistle' and mainly used in hepatic diseases. The anticancer efficacy of SLB is evident against various cancers in the last two decades, which were mainly through targeting proliferation, apoptosis, inflammation, angiogenesis and cancer cell metabolism. However, studies revealed that after oral administration of silibinin showed less bioavailability due to poor aqueous solubility. Recent advances indicated that silibinin encapsulated in nanocarriers like nanocrystals, nanoemulsions and liposomes improve its bioavailability and enhance site-specific and cytotoxic actions in cancerous cells. Magnetic nanoparticles (MNPs) are sub-microscopic solid particles which are composed of iron oxide and generally coated with polymers, silica and organic materials to ameliorate their colloidal stability and entrapment efficiency [4,5]. MNPs are preferred over other nanocarrier systems of anticancer drugs by virtue of their biocompatibility, chemical stability and easily modifiable property based on requirements of site-specific action and disease condition. MNPs act as a carrier system

for chemotherapeutic agents, nuclear acids, antibodies and radionuclide. MNPs combined with chemotherapeutic agents can simultaneously release drugs and selectively transfer heat to tumor cells. For better *in vivo* therapeutic applications, MNPs were fabricated with polymers to prevent aggregation in drug delivery systems. PLGA is a copolymer of lactic and glycolic acid widely used in the preparation of NPs due to its good biodegradability and biocompatibility. The aim of the present study was to prepare magnetic-core-based nanopolymeric carriers of anticancer drug, silibinin against kidney cancer cells (A-498) to potentiate cytotoxic action in comparison to free drug for determining the inhibitory effects. Primarily, magnetic nanoparticles (MNPs) were prepared as a magnetic core material, then encapsulated into PLGA using double emulsion method and further characterized in terms of particle size, zeta potential, % encapsulation efficiency, magnetic property, morphology, drug release behavior and cytotoxicity on kidney cancer cells (A-498). To confirm the safety and route of delivery, acute toxicity study was conducted by intravenous administration of magnetic-core-based silibinin nanopolymeric carriers (SLB-MPNPs) in *in vivo* study.

Graphene quantum dot (GQD) is an emerging class of nanocarbon material, exhibit photoluminescence property, excellent photostability and great biocompatibility with promising applications such as fluorescence imaging, biosensing and drug delivery. GQDs have lower cytotoxicity, large surface area and potential of surface functionalization that bring about more effective drug loading material [6–8]. Furthermore, the recent developments are carried on the combination of GQDs and nanomaterials for imaging and drug delivery. Magnetic nanoparticles (MNPs) especially iron oxide and its nanocomposite with GQDs, were applied in enhanced optical limiting, MRI (magnetic resonance imaging) and drug delivery. Loading of GQDs into MNPs are preferred over other nanomaterials as they were proven to be

excellent tumor diagnostic tools and treatment platforms and are utilized in various therapies such as magnetic hyperthermia and photothermal therapy. Polymeric nanoparticles have been tested to deliver wide range of drugs, such as small hydrophilic and hydrophobic drugs, vaccines, peptides, and biological macromolecules, via several routes of administration. Polylactic-co-glycolic acid (PLGA) is a biodegradable polymer extensively used in the formation of nanoparticulate carriers [9–11]. The delivery of actives with PLGA nanoparticles (PNPs) offers certain unique advantages like rapid drug release, lesser drug degradation, enhancement of drug bioavailability, higher rate of hydrolysis and lower toxicity. PNPs have a potential for influencing the mechanism of drug release and improving the bioavailability of poorly soluble drugs like curcumin, zoledronic acid, gentamicin sulfate, haloperidol, paclitaxel, vancomycin, estradiol, sparfloxacin etc [12–18]. The delivery of the drug might be site specific as the combination of MNPs and GQDs could be operated by magnetic fields. The present study was to develop magnetographene quantum dot nanocomposites (MGNs) with PNPs for delivery of SIN as fluorescent materials and assessed for particle size (PS), morphology, zeta potential (ZP), encapsulation efficiency (EE), magnetic property, fluorescence and drug releasing property. Also, cytotoxic activity, cellular uptake study and *in vivo* pharmacokinetic studies were also carried out.

However, SIN limits its applications due to less aqueous solubility (<50µg/mL), bioavailability (absolute oral bioavailability 95%) and extensive first-pass metabolism from the liver. Therefore, a promising approach is required to facilitate the application of SIN using encapsulation methods for improving physicochemical properties like solubility, particle size, surface charge, aggregation, stability, etc. [19,20]. 2-Hydroxypropyl–β-CD (HβD) is widely studied in drug inclusion complexation because of its higher inclusion functionality, better aqueous solubility and lower toxicity for

biological membranes. The incorporation of HβD inside the aqueous portion of lipid vesicles retains the liposome characteristics like membrane integrity and such system is termed as hybrid liposomes. Hybrid liposomes (HLs) are encapsulating carriers of a lipid-based particulate system to offer controlled release kinetics for a variety of poorly water-soluble drugs like risperidone, itraconazole, gemcitabine, curcumin, usnic acid, tretinoin, fluocinolone acetonide, essential oils like (*Lippia sidoides and Syzygium aromaticum*), etc. The present work was to develop inclusion complex of silibinin with HβD and further incorporated it into hybrid liposomes to improve solubility, bioavailability and stability as well as to explore the anticancer activity of silibinin on cell line A-498. The particle size, zeta potential, % encapsulation efficiency (% EE), *in-vitro* release and *in-vitro* cytotoxicity studies of the SIN formulations were assessed to check the suitability for anticancer activity in kidney cancer cell line (A-498).

Renal cancer (RC) is the most common form of kidney cancer and forms in the lining of renal tubules of the kidney. RC is most often discovered incidentally during abdominal imaging (ultrasonography, computed tomography, or magnetic resonance imaging) achieved for unrelated diagnostic purposes [1,21]. Therefore, when diagnosed, 25-30 % of patients are with metastatic disease. However, common analytical methods such as western blot or ELISA are complicated, expensive, time-consuming, therefore routine examination in clinical practice takes significant cost and time with sometimes inaccuracy in pathological results. Thus, predictable tumor markers as powerful tools for RC diagnosis and post-treatment surveillance. Recently, increased concentration of aquaporin-1 (AQP1) in patients with RC was identified as a definite and early marker of the disease. AQP1 is a water channel protein expressed in many epithelial tissues, endothelium and proximal tubule of the kidney. In addition, biomarker concentrations were not fundamentally altered by non-cancerous kidney

diseases like glomerulonephritis, diabetic nephropathy and urinary tract infection [22,23]. At present, there is no diagnostic technique for early detection of RC, other than incidental radiologic discovery. Biomarkers are readily measured substances that can be used to monitor normal and abnormal biologic function. Urine is the prototype non-invasive sample and is a useful biological fluid for biomarker analysis due to its ease of collection, availability of repeated samples and unlimited volumes [23–27]. Accurate and inexpensive, non-invasive methods of RC detection, using a urine as the substrate, remain a research priority. Biomarkers include genes, differences in protein expression and modifications of metabolite levels while several urinary biomarkers have been proposed as potential screening tools for cancer. The most promising urinary biomarker is AQP1, this biomarker can differentiate RC from healthy controls, benign renal masses and non-renal urological cancers. Urinalysis test strips provide an alternative method of cancer testing that is well-suited for screening purposes [23,28]. The median urine AQP1 level was 225.0 ng/mg urine creatinine in patients with confirmed RC versus 1.1 ng/mg in healthy individuals. A paper strip-based urine analysis system is well-suited for screening cancer patients because this biosensor changes color when reacting to a solution containing a biomarker for disease. The present research work emphasized on designing of paper strip-based non-invasive urine analysis system for qualitative detection of AQP1 biomarker in RC.

Rationale for selection of nanocarriers

Most of the herbal drugs limit the applications due to low aqueous solubility which lead to lower the bioavailability and consider the drug as a poor candidate for therapeutic application. However, the main reasons for limited bioavailability of silibinin are due to low aqueous solubility (<50µg/mL), poor penetration into the epithelial cells of intestine, high metabolism and rapid systemic elimination.

To overcome such problems, nanotechnology has shown great potential using different carriers like lipid nanoparticles, liposomes, nanocrystals, inclusion complexation with cyclodextrin, nanoemulsion, polymeric micelles and magnetic nanoparticles to improve the absorption of water insoluble drugs and considered as a promising approach to reduce side effects, enhance targeted and sustained drug release pattern.

The one part of research work aimed to develop biocompatible magnetic-core-based nanopolymeric carriers of poly (D, L-lactide-co-glycolic) acid (PLGA) encapsulated silibinin for the sustained release action on renal cancerous cell. The magnetic-core-based silibinin nanopolymeric carriers will act as a potential carrier for targeted transportation of actives in cancer therapy.

Another study focused on the characterization of magnetic graphene quantum dot nanocomposites for silibinin delivery with a specific interest in kidney cancer treatment optimized by a central composite design. Monodispersed magnetic graphene quantum dot nanocomposites are considered as an interesting alternative for silibinin delivery in case of kidney cancer.

The one part of research work focused on the development of inclusion complexes of silibinin with hydroxypropyl-β-cyclodextrin and further incorporated into hybrid liposomes to improve solubility, bioavailability and stability as well as to explore the anticancer activity of silibinin.

This drug delivery system exhibited higher cytotoxicity against kidney cancer cell line (A-498) through endocytic internalization into the cells and good pharmacokinetic properties as compared with silibinin. In near future, hybrid liposomes can be a promising carrier for increasing the solubility, bioavailability and stability of silibinin.

Rationale for selection of biosensor

At present, there is no diagnostic technique for early detection of renal cancer, other than incidental radiologic discovery.

A paper strip-based urine analysis system is well-suited for screening cancer patients because this biosensor changes color when reacting to a solution containing a biomarker for disease. The present research work emphasized on designing of paper strip-based non-invasive urine analysis system for qualitative detection of aquaporin biomarker in renal cancer.

Objective of work

Treatment (Drug delivery systems)

Magnetic polymeric nanoparticles

- Preparation of magnetic nanoparticles
- Preparation of silibinin-loaded magnetic PLGA nanoparticles (SLB-MPNPs)
- Characterization of nanoparticles
- Particle size and zeta potential
- % Encapsulation efficiency (% EE)
- ATR-FTIR spectroscopy
- Raman spectroscopy
- Morphology
- Vibrating Sample Magnetometry (VSM)
- *In vitro* release profile
- *In vitro* cytotoxicity study
- Stability study
- Acute toxicity study
- *In vivo* pharmacokinetics study
- Statistical Analysis

Magnetic graphene quantum dots

- Experimental approach of silibinin-loaded magnetic nanoparticles
- Fluorescent modification of SIN-mag-NPs
- Characterization of silibinin-loaded magnetic nanoparticles
- Particle size and zeta potential
- % Encapsulation efficiency (% EE)

- ATR-FTIR spectroscopy
- Morphology
- Vibrating Sample Magnetometry (VSM)
- X-ray fluorescence spectra
- *In vitro* release profile
- *In vitro* cytotoxicity study
- Stability study
- Acute toxicity study
- *In vivo* pharmacokinetics study
- Statistical Analysis

Drug-in-cyclodextrin liposomes

- Synthesis of inclusion complex
- Characterization of inclusion complex
- Saturation solubility study
- Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)
- X-ray diffraction (XRD)
- Differential scanning calorimetry (DSC)
- Proton nuclear magnetic resonance (^{1}HNMR)
- Scanning electron microscopy (SEM).
- Preparation of liposomes
- Characterization of liposomes
- Particle size, zeta potential and morphology of liposomes
- EE (%)
- *In vitro* release study

- *In vitro* cytotoxicity study
- Acute toxicity study
- *In vivo* pharmacokinetic study
- Storage stability of liposomes
- Statistical analysis

Diagnosis

- To develop urine based non-invasive biosensors for the treatment of renal cell carcinoma
- Preparation of dye-loaded cyclodextrin dispersion
- Preparation of paper strips
- Preparation of test samples
- Validation of the prepared paper strips
- Characterization of dye-loaded cyclodextrin dispersion
- ATR-FTIR spectroscopy
- Particle size
- Differential scanning calorimetry (DSC)
- SEM analysis
- Stability of dye-loaded cyclodextrin dispersion impregnated on Whatman filter paper

Materials and methods

Drug details

Procurement details of the SLB, are given in Table 1.

Table 1. Procurement details of the drug used in the formulation

Drug	Name of Supplier	Batch no.
SLB	Sigma-Aldrich, USA	BCBV3781

List of Excipients

The list of various excipients used in this project are listed in Table 2.

Table 2. Excipients used for execution of project work

Sr. No.	Name of Material	Name of Supplier
1.	Soya lecithin	Research lab Fine Chem Industries, Mumbai
2.	Cholesterol	Research lab Fine Chem Industries, Mumbai
3.	Resomer® RG 503 H, Poly (D,L-lactide-*co*-glycolide)	Evonik Industries, India
4.	Polyvinyl alcohol (PVA)	Sigma-Aldrich, India
5.	Hydroxypropyl–β-CD (HβD)	Roquette, Lestrem, France
6.	Tetrabromophenol blue (TBPB)	Sigma-Aldrich, India
7.	Pyromellitic dianhydride (PMDA)	Sigma-Aldrich, India
8.	β-cyclodextrin (β-CD)	Roquette, Lestrem, France
9.	Dimethylformamide (DMF)	Thermo *Fisher Scientific*, India
10.	Triethylamine (TEA)	Thermo *Fisher Scientific*, India

11.	Recombinant Human Aquaporin-1 (AQP1) biomarker	Recombinant Human Aquaporin-1 (AQP1) biomarker
12.	Qualitative filter paper (Whatman®; 125 mm diameter)	Sigma-Aldrich, India
13.	Graphene quantum dots	Sisco Research Laboratories Pvt. Ltd., Mumbai, India

Instruments

Sr. No	**Name of Instrument**	**Details**
1.	Analytical weighing balance	GR-200, A & D company Ltd., India
2.	High speed homogenizer	Remi Electrotechnik, India, RQT-127A/D
3.	Probe sonicator	Sonics, VCX 130, USA
4.	Rotary evaporator	IKA RV 10 digital, Staufen, Germany
5.	Ultracentrifuge	Beckman Coulter; Optim XPN-100, USA
6.	Malvern Zetasizer	Nano ZS90, UK
7.	UV-Visible spectrophotometer	Perkin Elmer, USA
8.	Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (*ATR-FTIR*)	PerkinElmer Instruments, USA
9.	Raman Spectrometer	Bruker BRAVO Raman, Australia
10.	High-resolution transmission electron microscopy (HRTEM)	Philips CM 200, Andover, Massachusetts, USA
11.	Vibrating sample magnetometer	VSM, Lake Shore 7410, USA

12.	Hematological and biochemical analyser	Celltac Alpha MEK-6500K, Japan and Erba Chem 7, Erba Mannheim, Germany
13.	Orbital shaker	Heidolph Polymax 1040, USA
14.	Differential scanning calorimeter	Mettler ToledoVR, Urdorf, Switzerland
15.	Bath sonicator	Oscar, Mumbai, India
16.	pH Meter	Universal enterprise, India
17.	Micropipette	Tarsons, India
18.	Vortex mixture	CM-101, Remi Equipment, India

Method of preparation

Preparation of magnetic nanoparticles

Accurately weighed 0.315 g of ferrous chloride was dissolved in 25 mL of distilled water and pH was adjusted to 7.8 using 1M KOH solution. Around 250 µL of 3% hydrogen peroxide solution was added in dark green solution of ferrous hydroxide for the formation of black precipitate. To collect iron oxide nanoparticles, the precipitate was separated with the help of magnet, washed with distilled water and dried in oven at 60-70 ºC [29]. This prepared formulation is abbreviated as MNPs. The preparation of iron oxide nanoparticles is shown in reactions 1 and 2 as follows:

$$2KOH + FeCl_2 = Fe(OH)_2 + 2KCl \text{ (Reaction 1)}$$

$$3Fe(OH)_2 + H_2O_2 = Fe_3O_4 + 4H_2O \text{ (Reaction 2)}$$

Preparation of silibinin-loaded magnetic PLGA nanoparticles (SLB-MPNPs)

SLB-MPNPs were prepared by double emulsion method (w/o/w) with minor modifications using sonication. PLGA was dissolved in 5 mL of acetone in test tube 1, whereas SLB (50 mg) was dissolved in 8 mL ethanol in test tube 2. Then 15 mg of MNPs and dissolved SLB (test tube 2 solution) were added to PLGA solution in test tube 1 and homogenized (Remi Electrotechnik, India, RQT-127A/D) at 8,000

rpm for 5 min. This primary emulsion was added to 10 mL of PVA (1%) aqueous solution and the mixture was sonicated (Probe sonicator, Sonics, VCX 130, USA) at 150W of energy output for 7 min. Further, acetone was allowed to vaporize using a rotary evaporator (IKA RV 10 digital, Staufen, Germany) and the NPs were purified by applying three cycles of centrifugation (10,000 rpm for 15 min, Beckman Coulter; Optim XPN-100 ultracentrifuge, USA). The supernatant was discarded and NPs were collected, dried and stored at ambient temperature.

The concentration of PLGA varied from 50-100 mg in SLB-MPNPs formulations as shown in table 3. Blank nanoparticles (MPNPs) were also prepared with the same method without using SLB.

Table 3. Composition of different formulations of SLB-MPNPs

Ingredient	**Formulation code**			
	M1	**M2**	**M3**	**M4**
SLB (mg)	50	50	50	50
PLGA (mg)	50	100	150	200
PVA (%)	1	1	1	1
MNPs (mg)	15	15	15	15

Preparation of silibinin-loaded magnetic nanoparticles

Solvent evaporation method was used to prepare silibinin-loaded magnetic nanoparticles (SIN-mag-NPs) with slight modifications [4,31]. SIN and PLGA were separately dissolved in organic solvents. 10 mg of MNPs and dissolved SIN were added in PLGA solution and homogenized at 8000 rpm for 3 min. This organic phase was added dropwise to a PVA solution, which was stirred at a rate of 8000 rpm using homogenizer for 5 min. The resulting emulsion was probe sonicated (SM-120PS, India) for 3 min. Then organic solvent was evaporated using a rotary evaporator under

reduced pressure and NPs were purified by centrifugation (10,000 rpm for 15 min, Thermo Sorvall Ultracentrifuge, MTX, USA). Two formulation criterions were optimized for their effects on the PS (Y1) and EE (Y2) of the developed NPs applying Design-Expert® Version 12 software. Based on formulation trials, PLGA (mg) (×1), PVA (%) (×2), and SIN content (mg) (×3) were considered as important variables affecting the preparation of NPs. Two parameters (PS and % EE) were examined at five different levels (− 1.782, − 1, 0, 1, 1.782) using a CCD (Table 1).

Fluorescent modification of SIN-mag-NPs

First 20 mg of optimised SIN-mag-NPs were incorporated into the solution of 3.2 mg N-hydroxysuccinimide (NHS) and 5 mg carbodiimide (EDC) in 10 mL distilled water. Further, 4 mL GQDs solution (2 mg/mL) was added into above solution and stirred at room temperature for 5 h. Finally, the obtained MGNs were collected by magnet, washed by acetone and dried.

Synthesis of inclusion complex

A homogenous paste of HβD was prepared in a mortar by adding 2 mL of ethanol. SIN was added to this paste, with continuous kneading for 30 min. An accurate amount of SIN (0.30 g) and HβD (0.93 g) were weighed in a molar ratio of 1:1. An appropriate quantity of ethanol was added further to maintain the suitable consistency of the paste and allowed to dry. This powdered mass passed through sieve number 120 and stored in airtight-containers until the further use.

Preparation of liposomes

Conventional liposomes comprising of blends of lipids soya lecithin and cholesterol were prepared by thin-layer evaporation technique in the weight ratios of 1:0, 1:0.5, 1:1 using a mixture of 2:1 v/v of chloroform: methanol and abbreviated as AT1, AT2 and AT3, respectively [32,33]. The organic solvents were removed under reduced pressure

in a rotary evaporator (Heidolph Laborota 4000 efficient, Germany) at a condition of 50^0C for 45 min to form a thin film on the inner surface of the round bottom flask. The film was hydrated by adding phosphate buffer saline (PBS) pH 7.4 then the liposomal suspension containing vesicles was kept under magnetic stirring for 5 min and further reduced the size in a bath sonicator (Oscar, Mumbai, India) for 15 or 45 s. Blank as well as drug-loaded liposomes were stored at 4°C until further analysis. To prepare silibinin-cyclodextrin-based liposomes (SCLs), SIN-HβD complex (0.55% w/v) was added in the aqueous phase. All other steps were used similarly to the original method.

Preparation of dye-loaded cyclodextrin dispersion

Accurately weighed 1.35 g of β-CD (1 mmol) and 0.004 g of TBPB were dissolved in acetone (9 mL) and DCM (3 mL). Subsequently, 2.1 g of PMDA (10 mmol) was added and magnetically stirred at 500 rpm for 15 min at room temperature to form a solution of dark green color. Further 5 mL of TEA solution was added gradually to get the stable blue-color. The obtained solid mass was ground with a mortar and pestle to obtain dye-loaded cyclodextrin dispersion.

Preparation of paper strips

Whatman® qualitative filter paper was used for the preparation of paper strips. The paper strips were immersed into the solution of cyclodextrin-based dye functional system for 1 min and kept for drying at room temperature, further stored (individually wrapped in aluminum foil) in a plastic container until further use.

Preparation of test samples

Artificial urine was prepared according to the procedure mentioned in Shmaefsky, et al. 1990 [34]. This biosensor was tested for various concentrations of AQP1 (10, 200, 400, 600, 800 and 1000 ng) present in artificial urine for color change.

Validation of the prepared paper strips

The calibration of the prepared paper strips was performed using various concentrations of AQP1 (10, 200, 400, 600, 800 and 1000 ng). The color changes were captured using the color grab smartphone application for HSV profiling. The calibration curves were plotted for AQP1 concentration versus change in H, S and V values, distinctly. This data was analysed in triplicates and with the standard deviations. Multiple measurements were taken for the estimation of specificity, repeatability and linearity of the prepared paper strips.

Characterization of nanoparticles

Particle size and zeta potential

The particle size and zeta potential of the formulations MNPs, MPNPs and SLB-MPNPs were measured by Malvern Zetasizer (Nano ZS90, UK) at 25^{o}C using distilled water to attain optimal scattering intensity.

% Encapsulation efficiency (% EE)

The amount of drug loaded in SLB-MPNPs was determined by indirect method. The nanoparticle suspension was centrifuged at 12,500 rpm for 45 min and the supernatant was analyzed for SLB content using UV-Visible spectrophotometry (Perkin Elmer, USA) at 288 nm.

The % EE was calculated by the encapsulated difference between the initial amount of SLB (S_T) and the amount of SLB remained in the supernatant (S_S), applying the following formula:

$$EE\ (\%) = [(S_T - S_S/S_T)] \text{ X } 100$$

ATR-FTIR spectroscopy

Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (*ATR-FTIR*) of SLB, PLGA polymer, MNPs, MPNPs and SLB-MPNPs were performed by UATR

spectrophotometer (PerkinElmer Instruments, USA). The scanning range was maintained between 450–4000 cm^{-1} with 32 cm^{-1} resolution.

Raman spectroscopy

Raman spectra were obtained by using portable Raman Spectrometer (Bruker BRAVO Raman, Australia) with a laser excitation wavelength of 514 nm and over the range of 400-3200 cm^{-1}.

Morphology

The morphological characteristics of MNPs and SLB-MPNPs were determined by using the high-resolution transmission electron microscopy (HRTEM) (Philips CM 200, Andover, Massachusetts, USA). The aqueous dispersion of NPs (5 µL) was placed on a copper grid followed by staining with 1% w/v phosphotungstic acid solution and allowed to air dry before imaging the particles under HRTEM.

Vibrating Sample Magnetometry (VSM)

The magnetic properties of MNPs and SLB-MPNPs were detected by vibrating sample magnetometer at ambient temperature (VSM, Lake Shore 7410, USA).

***In vitro* release profile**

In vitro release study of SLB from SLB-MPNPs was performed using a dialysis membrane (molecular weight cut-off 12,000 Da, Himedia Lab, Mumbai, India) with 30 mL of phosphate buffer (pH 7.4) at a stirring rate of 100 rpm and temperature of 37 ± 0.5^0C. The samples were removed at regular time intervals (1, 2, 4, 8, 24, 48, 90, 150, 180, 240 and 360 h), and were replaced with 2 mL of fresh phosphate buffer (pH 7.4). The aliquots were then filtered through 0.45 µm PVDF membrane filter (Millipore, USA) and analyzed on UV-Vis spectrophotometer at a wavelength of 288 nm for SLB. The reported values are average of three replicates (n = 3) with standard deviation.

***In vitro* cytotoxicity study**

In vitro cytotoxicity studies of the formulations such as SLB, PNPs, S-PNPs, C-PNPs and S-C-PNPs were studied to compare the effectiveness (Ochi et al. 2016; Karimpour et al. 2019). Human kidney cancer cell line A-498 were seeded in 96-well plates and treated with different concentrations (0.001-10 µg/mL) of the test formulations and incubated at 37°C, 5% CO_2 for 96 h. MTT reagent was added to the wells and incubated for 4 h, the formazan product formed by the cells was dissolved in DMSO and read at 550 nm on a plate reader. Measurements were performed and the concentration required for 50% inhibition (IC50) was determined.

Stability study

The formulation SLB-MPNPs was placed in a refrigerator at 4°C with a testing frequency of 0 day, 1 month, 2 months and 3 months. The particle size, zeta potential, % EE and *in vitro* release of SLB in NPs were considered as factors to evaluate stability of formulations. The supernatant obtained for % EE was used as per the procedure mentioned in the section 2.3.2.

Acute toxicity study

Adult female Swiss Albino mice (20–24 g) were maintained at 12/12 h light/dark cycle at room temperature of 25 ± 2^0C and relative humidity of 40–60%. All animals were kept for acclimatization for 7 days before carrying out the experiment. The study protocol was approved by Institutional Animal Ethics Committee (Approval number CPCSEA/IAEC/P-15/2019, dated 18th December 2019).

The acute toxicity of SLB-MPNPs was evaluated by *In-vivo* studies according to the previous reported method [36–38]. Adult female Swiss Albino mice (20–24 g) were randomly divided equally into two groups: one group with 5 mice received single *i.v.* administration of SLB-MPNPs (50 mg/kg) and another control group was treated with single *i.v.* route of same amount of normal saline solution. Animals were observed daily

for 14 days and special consideration was given for altered autonomic effects (salivation, lacrimation and piloerection), drowsiness, skin (fur), change in body weight, food, water consumption and mortality. Before sacrifice on 15 days, hematological and biochemical parameters were measured using Celltac Alpha MEK-6500K, Japan and biochemical analyzer (Erba Chem 7, Erba Mannheim, Germany). All animals were weighed and humanely sacrificed on 15^{th} day and internal organs of each animal were observed. The weights of heart, liver, spleen, brain, kidneys, lungs and stomach were noted and tissues were subsequently fixed, dehydrated, paraffin embedded, sliced and stained with hematoxylin-eosin for histological observation [38].

***In-vivo* pharmacokinetics study**

The Institutional Animal Ethics Committee (IAEC) approved the protocol with an approval number of CPCSEA/IAEC/PC15/2019. The animals (adult Swiss Albino mice weighing 22–25 g) were distributed into two groups, each contain 12 mice. The route of administration for SLB-MPNPs and SLB (50 mg/kg) was the intravenous route. At 0, 0.5, 1, 2, 4, 6, 10, 24 and 48 h post-dosing, approximately 0.5 mL blood samples were removed from the retro-orbital plexus into the heparinized centrifuge tubes. Plasma was prepared by centrifugation (8000 rpm × 10 min) at 4 °C and stored at −80 °C until analysis. The samples were analyzed through LCMS/MS (Shimadzu, LCMS-8040) following the procedure described by Pawar et al (2020) [39]. PK solver software was to determine pharmacokinetic parameters such as Cmax, area under the concentration-time curve from dosing time 0 to time t (AUC0-t), area under the concentration-time curve from time 0 extrapolated to infinite time (AUC0-∞), and MRT. Relative bioavailability (RB) for SLB-MPNPs against SLB drug solution was calculated as follows:

$RB = AUC_{SLB\text{-}MPNPs}/AUC_{SLB\ drug\ solution}$

Statistical Analysis

For statistical analysis, each of the experimental values were compared with its corresponding control group. The differences among treatment and control groups were analyzed by using GraphPad Prism V5.0 for Windows. p value < 0.05 was considered significant and results were stated as Mean ± Standard Error of Mean (SEM).

Characterization of inclusion complex

Saturation solubility study, Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR), X-ray diffraction (XRD), Differential scanning calorimetry (DSC), Proton nuclear magnetic resonance (^{1}HNMR) and Scanning electron microscopy (SEM) was performed for SIN, HβD and SIN-HβD complex.

To evaluate the solubility of SIN and SIN-HβD complex, a saturation solubility study was performed. Briefly, the excessive amounts of SIN and SIN-HβD complex were incorporated in 100 mL of distilled water and stirred at 200 rpm on an orbital shaker (Heidolph Polymax 1040, US) for 24 h. The dispersions were filtered and analyzed by a UV-Visible spectrophotometer (Perkin Elmer, USA) at a wavelength of 288 nm. ATR-FTIR study was used to examine the functional groups of SIN and SIN-HβD complex by UATR spectrophotometer (PerkinElmer Instruments, USA). The crystalline characteristics of SIN and SIN-HβD complex were determined by X-ray diffractometer (PANalytical) at 4°/min scan speed. The samples were analyzed at 2θ angle range of 5 - 60°. Accurately weighed samples of SIN, HβD and SIN-HβD complex were crimped into aluminum pans at 10 °C/min and recorded by DSC (Mettler ToledoVR, Urdorf, Switzerland) under nitrogen atmosphere. The ^{1}HNMR spectra of SIN and SIN-HβD complex were taken at 25 °C on a Magritek Spinsolve 60 (Carbon Model, Germany) operative at a proton frequency 60 MHz with 5 mm sample tubes and deuterated dimethyl sulfoxide was used as a solvent. Morphological characteristics of SIN, HβD and SIN-HβD complex formulation were examined using SEM (HitachiVR, Tokyo, Japan). In

SEM study, the samples were fixed on a brass stub using double-sided tape and made electrically conductive by coating with a thin layer of copper. The photographs were taken at an electric voltage of 20 kV and magnification factors of 500 and 2,500.

Characterization of dye-loaded cyclodextrin dispersion

The particle size of blank dispersion and dye-loaded cyclodextrin dispersion was determined using Nano ZS (Malvern Instruments, UK). The morphology of the herbosomal formulations was determined by TEM (JEM-Z300FSC, JEOL Ltd. Japan). ATR-FTIR study was used to examine the functional groups of β-CD, PMDA, TBPB, blank dispersion and dye-loaded cyclodextrin dispersion by UATR spectrophotometer (PerkinElmer Instruments, USA). Accurately weighed samples of β-CD, PMDA, TBPB, blank dispersion and dye-loaded cyclodextrin dispersion were crimped into aluminum pans at a 10^0C/min and recorded by DSC (Mettler ToledoVR, Urdorf, Switzerland) under nitrogen atmosphere. Morphological characteristics of dye-loaded cyclodextrin dispersion and dye-loaded cyclodextrin dispersion impregnated on Whatman filter paper were examined using SEM (HitachiVR, Tokyo, Japan). In SEM study, the samples were fixed on a brass stub using double-sided tape and made electrically conductive by coating with a thin layer of copper. The photographs were taken at an electric voltage of 20 kV and magnification factors of 500 and 2500.

Results and discussion

Preparation and characterization of MNPs and SLB-MPNPs

In the present study, double emulsion-solvent evaporation technique was used for the development of SLB-MPNPs where primary emulsion was prepared with change in concentrations of PLGA in organic phase from 50 to 200 mg, and the amount of drug and concentration of PVA were fixed to 50 mg and 1 %, respectively. It showed an average particle size of 206.4 ± 0.43 nm, whereas PdI of the formulation was 0.183. The zeta potential of this formulation was found to be -21.1 ± 0.76 mV which suggested stable

formulation. Formulation M3 was selected for further studies, based on particle size of 285.9 nm ± 0.28 and zeta potential of -14.71 ± 0.15 mV. The PDI values were all within 0.2 indicated the formation of homogenous NPs. The % EEies of the formulations were increased from 72.16 ± 1.06 % to 88.20 ± 0.15 % on increasing concentration of PLGA from 50 to 200 mg.

In the present study, double emulsion-solvent evaporation technique was used for the development of SLB-MPNPs where primary emulsion was prepared with change in concentrations of PLGA in organic phase from 50 to 200 mg, and the amount of drug and concentration of PVA were fixed to 50 mg and 1 %, respectively. The results in figure 6.3.1 showed that particle size directly proportional to the concentration of PLGA in the organic phase, it meant particle size increases from 228.6 to 372 nm as the concentration of PLGA increases from 50 to 200 mg. Besides, it was confirmed that the residual electrostatic charge was sufficient to declare the good stability of dispersions of drug-loaded NPs [40]. Formulation M3 of SLB-MPNPs exhibited relatively higher negative charges on the particles compared to formulation M1, which attributed to the carboxylic group of PLGA exposed on surface of the NPs. The possible effects of surface charge may affect the *In-vivo* life-span of the phytomedicines. The % EEies of the formulations showed the same order as that of particle size and polymer content which indicated the increase in particle size and polymer content increases the % EE [41,42]. Due to increase in particle size, the length of diffusional pathway of drugs from organic phase to aqueous phase increases with decrease in drug loss and increase in % EE [43,44]. It was also estimated that the uncapped PLGA exposed to a substantial amount of hydroxyl groups during the preparation and easily linked the components in SLB-MPNPs through hydrogen bond. Consequently, it was difficult for the SLB to leak out and increased the EE.

The particle size observed from HRTEM images was in accordance with that measured by Malvern Zetasizer, UK.

HRTEM images revealed the smooth and spherical shape of NPs without any aggregation but change in particle morphology of SLB-MPNPs as compared to MNPs, suggested the presence of SLB and MNPs. The dispersion of NPs was considerably improved and the dark-colored MNPs were present in the core of SLB-MPNPs.

The chemical structures of MNPs, SLB-MPNPs, PLGA polymer and drug were confirmed by ATR-FTIR spectroscopy. The appearance of absorption peaks at 3113.2 cm^{-1} and 1020.23 cm^{-1} are the characteristic bands for O-H and C-O-C bonds indicated the presence of ferric hydroxide in the MNPs [45,46]. The SLB spectrum showed characteristic principle peaks at 3455 cm^{-1} (-OH), 1166 cm^{-1} (C-C-C), 1269 cm^{-1} (C-O) and 824 cm^{-1} (C-H). The aromatic peaks were observed at 3561 cm^{-1} (-OH) and 1081 cm^{-1} (C-C-C) as reported [47,48]. FTIR spectrum of SLB-MPNPs showed a broad peak at 3300 cm^{-1} region and other peaks for SLB are present with smaller intensity, thus indicating that the drug was incorporated in NPs. ATR-FTIR spectroscopy indicated that the drug was completely incorporated in the MPNPs where strong chemical interaction was not found between SLB and other components of NPs, indicating compatible formulation [49].

Raman spectra were recorded for the identification of MNPs and demonstration of the interactions involved in the formation of the SLB-MPNPs based on the significant spectral changes of free SLB and SLB-MPNPs. The strongest band at 494 cm^{-1} corresponds to A1 g mode of Fe_3O_4 which was observed in the spectrum of SLB-MPNPs. The spectrum collected in the present investigation may also show a weak band around 543 cm^{-1} in SLB-MPNPs, attributed to the scattering of Eg mode of Fe_3O_4, however, this band can be absent in case of SLB. The characteristic bands to SLB positioned at 3066

cm^{-1}, 2879 cm^{-1}, 1614 cm^{-1}, 1166 cm^{-1} and 703 cm^{-1} were observed in SLB-MPNPs but with smaller intensity, indicating successful encapsulation of SLB into SLB-MPNPs. Raman spectroscopy confirmed the formation of MNPs and based on the changes in the significant spectra of SLB and SLB-MPNPs, the successful loading of SLB into SLB-MPNPs was observed [50].

The saturation magnetization of MNPs in the hysteresis curve of 36.35 emu/g which was higher in comparison to the saturation magnetization of SLB-MPNPs (12.78 emu/g). In VSM analysis, changes in the saturation magnetization of MNPs and SLB-MPNPs indicated that the encapsulation of MNPs and SLB within the PLGA polymer relates to the increase of coating layer and the decrease the mass percentage of iron oxide. When the external magnetic field was removed, both the formulations were well dispersed by gentle shaking.

***In-vitro* cytotoxicity study**

At all tested concentrations, SLB-MPNPs showed higher cytotoxicity as compared to SLB. The IC50 of SLB-MPNPs was 3 ± 1.76 µg/mL, whereas free SLB showed higher IC50 value 5 ± 0.91 µg/mL on A-498 human kidney cancer cells. *In-vitro* cytotoxicity studies were performed to evaluate the anti-tumor potential of formulation SLB-MPNPs and SLB at equivalent concentrations of drug. Anti-tumor effect of anticancer drug, SLB increased with the use of iron oxide nanoparticles through endocytic internalization into the cells. In such a delivery system, the synergy of magnetic fields and anticancer magneto-sensitive nanoparticle led to increase in anti-tumor activity.

***In-vivo* pharmacokinetics study**

The mean plasma-drug concentration-time curve demonstrated a comparable area under the curve, i. e., AUC0-∞ = 108.89 for SLB-MPNPs and AUC0-∞ = 44.09 for SLB, respectively. With respect to SLB-MPNPs, the initial drug concentration noticed after

0.5 h was 7.3 ± 21.56 µg/mL which increased with a Cmax and Tmax of 8.24 ± 17.89 µg/mL and 1 h, furthermore decreasing to a final concentration of 0.75 ± 3.9 µg/mL after 48 h. The clearance of SLB-MPNPs was 7.57 mL/h and of free SIN was 39.00 mL/h. Also, the mean residence time (MRT) of SLB-MPNPs in the plasma was 32.86 h, 6.53 times that of free SLB. Overall, SLB-MPNPs had the longer $t_{1/2}$ and MRT, lower CL than the free drug in mice. The sustained release of SLB from SLB-MPNPs in the blood confirmed that the bioavailability of SLB increased when it was loaded into SLB-MPNPs.

***In-vivo* acute toxicity of SLB-MPNPs**

To determine the safety of SLB-MPNPs as a drug delivery system, the *In-vivo* acute toxicity study was performed. Throughout the observation period, no animals showed any abnormality or behavioral changes whereas tremors, convulsion, salivation, diarrhea and lethargy were also not observed. The data of hematological parameters, biochemical parameters and organ weights for female mice between the control and experiment groups did not show significant difference Histopathological studies were carried out to observe the effect of SLB-MPNPs on the vital organs like liver, kidney, spleen, heart, brain, lung and stomach. In case of liver, the centrilobular area and hepatocytes were normal in both the groups. The heart showed normal myocardial muscle fibers, whereas unremarkable renal tubules and glomeruli were observed in case of kidneys. The spleen was not affected by SLB-MPNPs as normal red and white pulp were observed. In case of brain, neuronal cells and cerebral parenchyma were observed without significant changes, whereas in lungs, the alveolar structures were normal between two studied groups. No significant toxicity was observed in the experimental animals after *i.v.* administration of SLB-MPNPs at a dose of 50 mg/kg. Thus, the acute toxicity indicated that safety of SLB-MPNPs as a drug delivery system.

Stability study

SLB-MPNPs formulation was stored at 4 °C and protected from direct light for stability studies. Particle size, zeta potential and % EE revealed insignificant changes during the stability study period. Also, *In-vitro* release study for 0 month, 1 month, 2 months and 3 months storage samples of SLB-MPNPs showed no substantial changes, which suggested the stable formulations.

In this study, EE (Y2) of NPs was obtained in the range of 62 – 92 %. The R^2 value for the model was found to be 0.8938. This actual R^2 value was greater than the adjusted R^2 value (0.9176) and all selected parameters were significant at $p \leq 0.05$.

Characterization of SIN-MGNs

The TEM images of the GQDs and SIN-MGNs represented spherical shape of NPs with good dispersibility. The absorption band examined at 546.39 cm^{-1} confirming the presence of Fe–O group [45]. The SIN spectrum presented distinctive peaks at 1269 cm^{-1} (C-O), 1166 cm^{-1} (C-C-C) and 3455 cm^{-1} (-OH). SIN-MGNs spectrum displayed a broad peak at 3300 cm^{-1} and new peaks that are mentioned for SIN, SIN-mag-NPs and GQDs were present with smaller intensities, this showed that the drug was incorporated in the SIN-MGNs.

It was shown that 36.05 ± 0.36 % of SIN released till 24 h and drug release improved and the cumulative amount of SIN release over 2 days was 45.16 ± 0.5 %, the SIN release profile exhibited a sustained release pattern. In PBS, PLGA could be biodegraded by hydrolysis of ester linkage between them. So the much slower release from SIN-MGNs after 48 h could be attributed to slow diffusion of drug from the NPs interior, by the degradation of PLGA.

SIN-MGNs exhibited higher cytotoxicity as compared to GQDs, at all tested concentrations. The IC50 values of GQDs and SIN-MGNs on A-498 cells were <10 ±

0.14 µg/mL, 6 ± 1.37 µg/mL respectively. The preferential cytotoxicity of SIN-MGNs on A-498 cells can be attributed to the specificity of SIN to aberrantly expressed molecular targets in renal cancer cells. Several studies have shown anticancer activity of SIN on A-498 cells. Through either SIN inhibiting cellular growth via the up-regulation of insulin-like growth factor binding protein 3 (IGFBP-3), or by inhibiting EGFR (epidermal growth factor receptor) signal-induced migration and invasion of renal cancer cells.

Drug-inclusion complex

Characterization of inclusion complex

The saturation solubility of SIN was found to be 37.22 mg/mL whereas in the HβD complex was 73.95 mg/mL. The solubility studies suggested that SIN was poorly soluble in distilled water but due to the incorporation of SIN into the inner structural cavity of HβD improved the solubility of SIN as reported. SIN demonstrated irregularly shaped crystals to form aggregates while HβD consisted of spherical and intact shaped particles. The complex showed particles of HβD embedded with SIN particles and comparable morphology with pure compounds. SEM images revealed a comparable morphology between SIN and SIN-HβD. After the complexation process and grinding, the HβD and SIN particles showed a complex structure because of small particles of SIN attached on the surface of HβD particles in the aggregated form as shown in Figure 6.8.2c [51].

SIN showed distinctive peaks at 3455 cm^{-1} (-OH), 1269 cm^{-1}(C-O), 1166 cm^{-1} (C-C-C) and 824 cm^{-1} (C-H). The aromatic peaks at 1081 cm^{-1} (C-C-C) and 3561 cm^{-1} (-OH) were shifted because these groups were involved in the formation of hydrogen bonds associated with the entrapment of the aromatic ring of the SIN within the hydrophobic cavity of HβD [52]. The modifications were possible due to the controlled vibration of SIN complexation with HβD [19].

The thermal curve of SIN showed a sharp melting endothermic peak at 170°C corresponding to its melting point. The DSC thermogram of HβD confirmed a broad endotherm in the range of 39-72°C. In the thermal curve of the SIN-HβD complex, the endothermic peak changed into a slightly broadened peak [53]. DSC study suggested a strong interaction of amorphous entity in addition to the interaction between SIN and HβD [54].

The XRD pattern of SIN was characterized by the presence of sharp peaks at 13.63, 17.63, 16.02, 17.57, 22.35, 24.53 and 31.65 (2θ) illuminating the crystalline nature of the drug. The crystalline nature of HβD was confirmed from the presence of sharp peaks at 8.98, 10.27, 10.7 and 19.02. The diffractogram of SIN-HβD complex was found to be without characteristic peaks at 14.76, 16.21, 17.44, 20.80, 22.43, 24.64, 26.45 and 32.43 of drug indicated loss of its crystalline nature. In the case of XRD study, the number of sharp peaks existing with the drug was found to be decreased in the case of SIN-HβD complex due to the formation of inclusion complex [55].

^{1}H NMR images SIN and SIN-HβD complex showed that an upfield shift for the protons (H3 and H5) of SIN in the presence of HβD indicated the complexation of SIN and HβD. Both terminal aromatic portions of SIN are included in the hydrophobic cavity of HβD. The two condensed rings of SIN were incorporated in the lipophilic segment of HβD while the aromatic ring bearing a free phenolic hydroxyl and methylated at ortho-position interacts with the β-hydroxypropyl group of the HβD as well as with H2 and H4 protons [51,56].

Characterization of conventional and hybrid liposomes

According to characterization parameters of conventional liposomes, liposome containing soy lecithin and cholesterol of 1:1 ratio (AT3) was selected for the preparation of HLs. The particle size distribution of liposomal formulations was

determined by the photon correlation spectroscopy technique and found in the range of 200 – 500 nm. The PDI values were found to be within 0.2 indicating the formation of homogenous liposomes. The zeta potential of all the formulations was found to be more than -30 mV which indicated stability. The % EEs of the formulations AT1, AT2, AT3 and HLs were found to be 84.28 ± 1.06 %, 85.86 ± 1.69 %, 85.79 ± 0.47 % and 92.06 ± 0.39 %, respectively. It confirmed that the shape of HLs was homogenous, spherical and unaffected by the incorporation of HβD [57]. The *In-vitro* drug release patterns of both the formulations are displayed in Figure 6.8.8. The rapid release of drug from HLs compared with conventional liposomes was confirmed because of the interaction between HβD and phospholipid's bilayers of HLs, resulting in partial leakage of drug from vesicular structures [58].

Impregnation of dye-conjugated miniaturized system on paper strip for detection of biomarker in renal cancer

ATR-FTIR spectroscopy

ATR-FTIR spectra were obtained to evaluate characteristic groups of β-CD, PMDA, TBPB, blank dispersion and dye-loaded cyclodextrin dispersion. As shown in figure 6.9.1, the spectrum of β-CD showed a featured peak at 1024.39 cm^{-1}, which was attributed to the stretching vibration of C-O-C groups. The absorption bands at 3394 cm^{-1} and 1703 cm^{-1} were attributed to stretching vibrations of O-H and C=O groups, respectively. In case of TBPB, absorption peak at 3133.56 cm^{-1}, 2900 cm^{-1}, 1372 cm^{-1} and 690-528 cm^{-1} were attributed to the stretching vibrations of OH, C-H and C-Br groups respectively. Small but significant shifts of peaks and discrete variations of their relative intensities were observed for the blank and dye-loaded cyclodextrin dispersion, suggesting a host-guest interaction.

Particle size

The particle size of dye-loaded dispersion was found to be 1034.34 ± 3.75 nm which was larger than that of blank dispersion (896.78 ± 2.54 nm). The larger particle size of dye-loaded cyclodextrin dispersion is due to the interaction of TBPB and β-CD.

Differential scanning calorimetry (DSC)

DSC analysis provides information about the crystallinity and interactions of sample. As shown in figure 6.9.2. TBPB showed an endothermic melting peak at 234.81^{0}C and PMDA exhibited endothermic melting at 285.19^{0}C. The wide peak at 219.49°C, which is observed for β-CD, shifts to 254.49°C in the dye-loaded dispersion, indicating that the developed system does not contain much residue of TBPB or β-CD, thus suggesting that the dye is well dispersed in the cyclodextrin cavity.

The DSC thermograms showed the differences in melting points. This is due to the thermal transition behaviour of dye-loaded dispersion. The modifications were possible due to the controlled vibration of TBPB complexation with β-CD.

SEM analysis

β-CD is widely studied in drug inclusion complexation using higher inclusion functionality and aqueous solubility. In this work, the reaction of β-CD with TBPB was achieved to expedite a sensitive reaction in the existence of AQP1 biomarker. Figure 6.9.4 shows the visual color changes of the paper strips prepared with cyclodextrin-based dye functional system in response to the AQP1 concentration of the artificial urine samples. It is observed that the test strip dipped into a sample, yellow color intensity increased with a decrease in AQP1 concentration. The results confirmed that the prepared paper strips had a good response to the change in AQP1 concentration (10–1000 ng).

This HSV profiling was carried out for color analysis of this cyclodextrin-based paper strip. The paper strip demonstrated linearity, reproducibility and specificity to AQP1

detection with limit of detection (LOD) of 261.19 ng and limit of quantification (LOQ) of 791.49 ng within a response time of 15 sec.

The paper strip changes color due to decrease in pH upon the interaction with AQP1 biomarker present in artificial urine samples. The color change during the interaction might be due to the transformation of dye system of free basic form into bound acidic form. The lower the concentration of AQP1 present in the sample, the color change was more reflective. The observation is not affected by high concentrations of interfering proteins such as albumin and alterations in urine pH, due to high sensitivity with AQP1 biomarker. β-CD is commonly used to increase solubility of guest molecule hence binding of TBPB and AQP1 increases, it is sensitive at lower concentration of AQP1. Interaction between β-CD and TBPB with PMDA crosslinker increases the uptake of the dye. The marked improvement of dye uptake considering the encapsulation of TBPB in the β-CD cavities or covalent bonding with the PMDA/β-CD network. Therefore, with the hydroxyl group of TBPB, it is not feasible to form intermolecular hydrogen-bond interactions with β-CD but it forms a hydrogen-bond interaction with the Br atom of TBPB, giving a host-guest interaction.

Stability of dye-loaded cyclodextrin dispersion impregnated on Whatman filter paper

The dye-loaded cyclodextrin dispersion impregnated on Whatman filter paper strips must be protected from environmental air exposure during storage and individually wrapped in aluminum foil in a plastic container with a moisture-absorbent material like silica for 3 months. No change in color reported during the stability study period and indicated stable paper strip.

Conclusion

Silibinin offers potential anticancer effect with less aqueous solubility and high permeability. Magnetic-core-based silibinin nanopolymeric carriers were prepared with the double emulsion solvent evaporation method for the treatment of renal cell cancer. The newly developed drug delivery system possessed an acceptable particle size, zeta potential and high % EE with a greater inhibitory effect on the growth of kidney cancer cells (A-498) as compared to free silibinin. Stability studies indicated that the storage of SLB-MPNPs at refrigerated conditions increases the stability of the formulation for a period of 3 months. Acute toxicity study revealed that SLB-MPNPs were found to be safe at a dose of 50 mg/kg for administration in mice. Silibinin is a clinically accepted herbal drug with minimum side effects, therefore the magnetic-core-based silibinin nanopolymeric carriers considered as an effective targeted drug delivery system for treating renal cell cancer.

The present study focused on the characterization of magnetic graphene quantum dot nanocomposites for silibinin delivery with a specific interest in kidney cancer treatment optimized by a central composite design. It revealed that particle size was below 300 nm, high encapsulation efficiency (81.27 %) and cumulative *in-vitro* drug release in sustained manner. *In-vitro* cellular uptake studies with renal cancer cells (A-498) demonstrated that the magnetic graphene quantum dot nanocomposites presented higher cellular uptake of silibinin and cytotoxicity than graphene quantum dots. The *in-vivo* pharmacokinetic study achieved a significant improvement in the drug bioavailability and increased mean residence time specified the prolonged existence of silibinin in systemic circulation. Therefore, monodispersed magnetic graphene quantum dot nanocomposites are considered as an interesting alternative for silibinin delivery in case of kidney cancer.

Cyclodextrin-based liposomes were prepared using hydration of thin lipid film method with subsequent sonication process. Molecular docking studies projected the formation of complex; at the same time, the results obtained from ATR-FTIR, DSC, XRD, ^{1}HNMR and SEM studies indicated that stable silibinin-hydroxypropyl-*β*-cyclodextrin complex was prepared at a molar ratio of 1:1. The particle size distribution of liposomal formulations was obtained in the range of 200 – 500 nm, the zeta potential was found to be more than -30 mV and release of silibinin from liposomes was maximum (more than 80 %) when the drug was encapsulated in the form of a complex with hydroxypropyl-*β*-cyclodextrin for better solubility. This drug delivery system exhibited higher cytotoxicity against cell line (A-498) through endocytic internalization into the cells and good pharmacokinetic properties as compared with silibinin. In near future, cyclodextrin-based liposomes can be a promising carrier for increasing the encapsulation efficiency and stability of silibinin.

A non-invasive paper strip-based biosensor was developed for the estimation of AQP1 levels in artificial urine samples. The sensor was prepared by paper strip impregnated with cyclodextrin-based dye functional system and color change corresponding to AQP1 concentration was determined by HSV profiling based on slope method. This paper strip showed high sensitivity and appropriate tool to detect AQP1 concentration in urine qualitatively. The fabricated biosensor displayed good linearity to detect AQP1 which indicated that the development of point-of-care, novel, personalized diagnostic and treatment protocol. In future, the mobile application software can be programmed for the determination of S values and storage of data to recognize renal cancer qualitatively using AQP1 biomarker present in urine samples.

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